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TRANSGENIC (T_1) TOBACCO (*Nicotiana tabacum*) PLANTS OVER EXPRESSING FERRITIN SHOWED TOLERANCE AGAINST OXIDATIVE STRESS

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ABSTRACT

Among different types of Reactive oxygen species, hydroxyl radicle (OH^-) is the most toxic and dangerous to the cell and is generated by iron mediated Fenton reaction. Ferritin is a key protein in the iron homeostasis and sequesters iron and makes it non available freely to mediate formation of hydroxyl radicle. With this view, transgenic plants overexpressing ferritin were developed and in the present study T_0 seeds obtained from putative transformants were screened on kanamycin for the presence of ferritin gene. Presence of ferritin gene in T_1 plants is confirmed by PCR analysis and further leaf discs were exposed to Methyl Viologen induced oxidative stress followed by high light stress to confirm the role of ferritin in oxidative stress. The results indicated that the percent electrolyte leakage and cell damage was higher in wild type plants as compared to transgenic plants. The transgenic plants grew taller and started flowering earlier as compared to wild type plants. Our results indicated that the over expression of ferritin in chloroplast gives tolerance against oxidative stress as compared to wild type plants both at seedling level and at the plant level.

KEYWORDS: Oxidative Stress, Ferritin, Iron, Methyl Viologen and Hydroxyl Radicle

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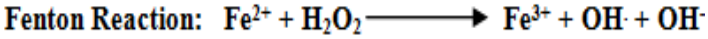
Original Article

INTRODUCTION

Almost all these abiotic stresses triggers the generation of reactive oxygen species (ROS) leading to oxidative stress. Formation of singlet oxygen subsequently stimulates production of other ROS such as hydrogen peroxide (H_2O_2), super oxide anion (O_2^-) and hydroxyl radicals (OH^-). These reactive molecules are highly destructive to lipids, nucleic acids, and proteins. Among them (OH^-) is the most toxic and dangerous.

To minimize the damage by ROS, plants have evolved certain defense mechanism. The defense systems include non-enzymatic and enzymatic antioxidants. All the antioxidants are mainly involved in scavenging H_2O_2 and O_2^- radicals, H_2O_2 and O_2^- by themselves are relatively less damaging, but they can form species which can damage the essential cellular components such as hydroxyl radicals (OH^-) that can initiate lipid peroxidation and also attack DNA, proteins and many small molecules. Once OH^- is formed, it is very difficult to manage and no antioxidants are present in plants. So, prevention of its formation gains importance.

Ferritin: An iron storage protein, widely distributed in the living organisms have been implicated to play an important role in the oxidative stress. The major role of ferritin in reducing oxidative stress seems to be by regulating the free iron levels in the cell.



Iron is toxic to cell when it is free because it acts as catalyst in the production of OH^- radicals through Fenton reaction (Arora *et al*., 2002, Theil *et al.,* 2006). This opens up an option to manage OH^- radicals by regulating the ferritin in the plant systems (Udaya Kumar *et al.,* 1999).

With this background, the transgenic tobacco plants overexpressing ferritin gene were developed and they were confirmed for presence of ferritin protein and its tolerance under oxidative stress. In the present study to confirm the role of ferritin in oxidative stress tolerance, the T_1 transgenic tobacco plants overexpressing ferritin gene is screened for its performance under different levels of oxidative stress.

MATERIALS AND METHODS

The study was to analyze the T_1 seeds of the primary Ferritin transgenics developed earlier in the laboratory by transferring *pFer/Rok2* construct into the tobacco wild type plants. The gene construct harbored the full-length cDNA of Ferritin from alfalfa under the influence of *CaMV* promoter and it is targeted into chloroplast. It has kanamycin as both bacterial and plant selectable marker. The putative transgenic plants obtained were confirmed by PCR analysis using *npt II* (which codes for kanamycin resistance) primers and by southern blot analysis. The transgenic plants showed higher tolerance to oxidative stress compared to wild type. Out of the several primary transformants, one of the primary transformants was selected for the present study.

- **Screening of T_1 Seeds on Kanamycin Selection**

The seeds collected from wild type and the Ferritin primary transgenic plants (T_0) were pretreated with 200 ppm GA for 24 hours in order to facilitate proper germination. The seeds were germinated on water for two days. The germinated seedlings were transferred to 500ppm kanamycin. The percentage seedling survival on kanamycin was recorded four days after incubation. Later the seedlings were exposed to fluorescent light for one day. The seedlings remained green were considered as transformed. Based on this segregating pattern was computed.

- **Screening of Transformants (Kanamycin Resistant) for the Methyl Viologen (MV) Induced Oxidative Stress**

Wild type seedlings and transgenic seedlings surviving on the 150 ppm of kanamycin were selected and the seedlings were spread on pertriplate containing different concentrations of MV (5, 10, 15, 20, 25 µM). Then the plates were exposed to low light (200 µ Ein/m^2/sec) for 8 hours for MV uptake and further they were exposed to highlight (1500 µ Ein/m^2/sec) for 3 hours. Then the plates were kept for recovery in dark for 12 hours and percent survival of seedlings was recorded. The seedlings, which survived on 25 µM of MV, were then transferred to small plastic cups containing soilrite and kept in the normal laboratory conditions for 25 days and then they were transferred to big battery containers in the field. After 20 days the experiments were carried out in these plants.

- **Molecular Analysis of T_1 Transgenic Plants Selected on Kanamycin and MV.**

Transgenic Tobacco plants established in the field were analyzed for the presence of ferritin gene by PCR analysis by using Forward primer (5` CGA GAG CGA GTT TTT GGT A 3`) and Reverse primers (5` TTG CGG GAC TCT AAT CAT AA 3`). On amplification the expected product should be 556 bp.

The PCR product of transformed, wild type and plasmid (*pFer/Rok2*) was checked for the presence of the gene by agarose gel electrophoresis. The bands in the gel was then viewed on a transilluminator and documented with the help of gel-documentation system.

PCR positive Ferritin plants (T_1 plants) were assessed for relative oxidative stress tolerance. The 25-30 days old plants maintained in pots under greenhouse condition were used for physiological studies for its resistance to oxidative stress at plant level.

- **Membrane Integrity Test**

MV is a systemic herbicide (Paraquat), its application on plant cells leads to the creation of ROS. It blocks the electron transfer from ferredoxin to NADP in the Photosynthetic electron transport chain. So, it is used for conducting the experiments related to oxidative stress in the laboratory conditions. The leaf discs of transformed and wild type plants were floated on 2µM and 5µM concentration of MV and water.

The plates were incubated for five hours in dark. This period of incubation facilitates the entry of the MV into the cells. Then the plates were exposed to high light intensity of 1500 µ Ein/m^2/sec for three hours. This high light intensity would be sufficient to enhance the generation of the free radicals. The light exposed leaf disc were then transferred to dark and allowed for recovery for 8 hours. During this recovery process the damaged cells leak out the cell content into the solution. After 8 hours the total leachates were collected and the electrical conductivity for inorganic ions was measured by the Electrolyte Conductivity meter EC (EC-CD analyzer, CM-183) (Leopald, 1981, Gonger *et al.*, 2013).

- **TTC Test for MV Induced Oxidative Stress**

The cell viability of MV treated leaf discs was assessed using property of TTC reduction by respiratory enzymes, which converts colorless TTC to red Formazon. The red color was measured at 485 nm. The red color intensity is an index of chlorophyll activity and cell viability. Leaf discs from T_1 Ferritin transformants and wild type plants were incubated in MV (2 µM and 5 µM) and water for 5 hours and then exposed to highlight (1500µ Ein/m^2/sec) for 3 hours. Further, the leaf discs were kept in dark for 8 hours recovery. The recovered leaf discs were then incubated in 5 ml of 0.4% TTC for 2 Hours. TTC was decanted and the leaf discs were washed with distilled water 5 ml of 2-Methoxy ethanol was added to the leaf discs and dried on water bath to dryness. Again 5 ml of 2-methoxy ethanol was added and the absorbance was taken at 485nm. Percent reduction of cell viability over control was calculated (Belcher, 1975, Gonger *et al.*, 2013).

- **Growth Parameters**

The plant height of the transgenic and wild type plants was recorded on 40^{th} day after transferring to battery pots in the green house. The flowering date of both transgenic and control plants were recorded. Then these flowers were bagged to facilitate the self-pollination and after maturity the seeds were collected and stored.

RESULTS AND DISCUSSIONS

- **Screening The T_1 Seeds on Kanamycin and Further on MV Induced Oxidative Stress**

After 3 days of incubation, 82% seeds showed germination, but none of the wild type seeds germinated on kanamycin. In water both wild type and the transgenic seeds showed 100 percent germination. This initially confirms the

integration of *npt II* gene into the genomic DNA and it also indicated the normal segregation ratio of 3:1 (Table 1). Wild type seedlings could survive upto 15μM MV, whereas T_1 transgenic seedlings could survive even on 25μM MV (Table 2).

Table 1: Effect of Different Concentrations of kanamycin on Percent Germination of T_1 Ferritin and wild Type Seeds

Kanamycin Concentration(ppm)	Germination %	
	Wild Type Seeds	T_1 Ferritin Transgenic Seeds
0	100	100
150	0	82

Table 2: Effect of Methyl Viologen on the Percent Survivability of T_1 ferritin and wild Type Seedlings

Methyl Viologen Concentration (μM)	% Seedling Survival	
	T_1 Ferritin Seedlings	Wild Type Seedlings
5 μ M	100	100
10 μ M	100	80
15 μ M	79	3
20 μ M	50	2
25 μ M	15	0

- **Molecular Analysis of the T_1 Transgenic Plants Selected on Kanamycin And MV**

The agarose gel indicates the presence of 556 bp Ferritin fragment in the T_1 ferritin transgenic plants and also in the *pFer/Rok2* plasmid DNA. But, the band was not present in the wild type plants (Figure 1). The results suggest that all the *npt II* positive putative plants selected for PCR analysis with ferritin gene specific primers were ferritin positives.

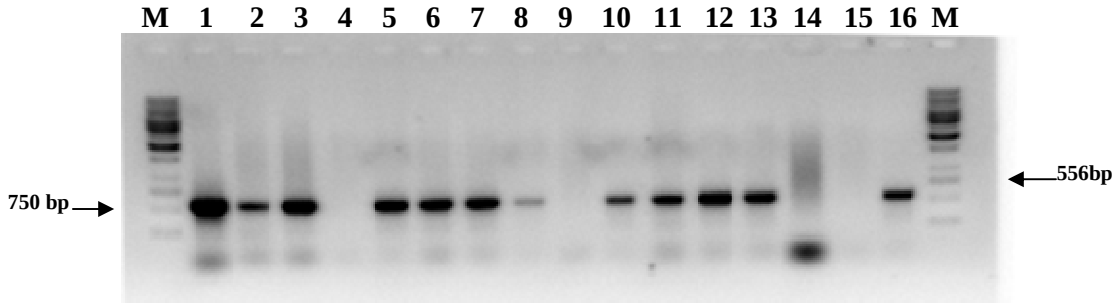


Figure 1: Agarose Gel showing the PCR Amplified Products for Ferritin Gene Specific Primers

M : Gene ruler 1kb ladder, Lane 1 : plasmid DNA from pFer/Rok2 construct, Lane 2,3,5,6,7,8,10, 11,12,13,and 16 : T_1 Ferritin plants, Lane 4,9,14,15 : Wild type plants. The genomic DNA isolated from wild type and T1 transgenic tobacco plants were subjected to PCR analysis using ferritin gene specific primers. The PCR products were analyzed on the 1.2% agarose gel. The amplified product of 556 bp was obtained in plasmid (Lane: 1), and T_1 ferritin PCR positive plants (lane 2,3,5,6,7, 8,10,11,12,13, and16), but band was not seen in T_1 ferritin PCR negative plants (lane 4,9,14,15) and in wild type tobacco plants.

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- **Membrane Integrity Test**

The results indicated that the percent electrolyte leakage was 62 and 44 per cent in wild type and transgenic plants at 2 µM and at 5 µM it was 88 and 50 percent in wild type and transgenic plants respectively (Figure 2). The loss in membrane integrity was high in wild type plants and low in Ferritin transgenic plants under MV induced oxidative stress.

- **Cell Viability Test**

At 2 µM of MV, percent reduction in cell viability over control was 27.2 in wild type and 14.8 in transgenic plants. At 5 µM, 33.5 percent reduction in cell viability was seen in wild type plants and 28.6 percent in transgenic plants. The results indicated that the transgenic plants maintained higher cell viability at both MV concentrations compared to wild type plants (Figure 3). Ferritin plays a major role in regulating free iron levels and thus prevents the excess iron mediated Fenton reaction, which otherwise leads to the formation of toxic free radicals. The results clearly indicates that ferritins are not likely to be an essential iron source for plant development, but that they play a significant role in the defence machinery against oxidative stress (Ravet *et al.*, 2009).

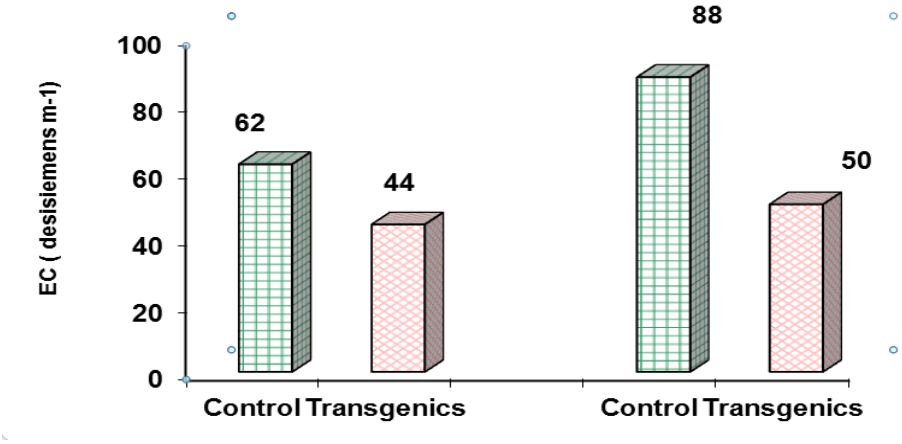

Figure 2: The Electrolyte leakage in wild type and transgenics overexpressing *Ferritin* under Methyl Viologen (2 µM and 5 µM**) induced oxidative Stress**

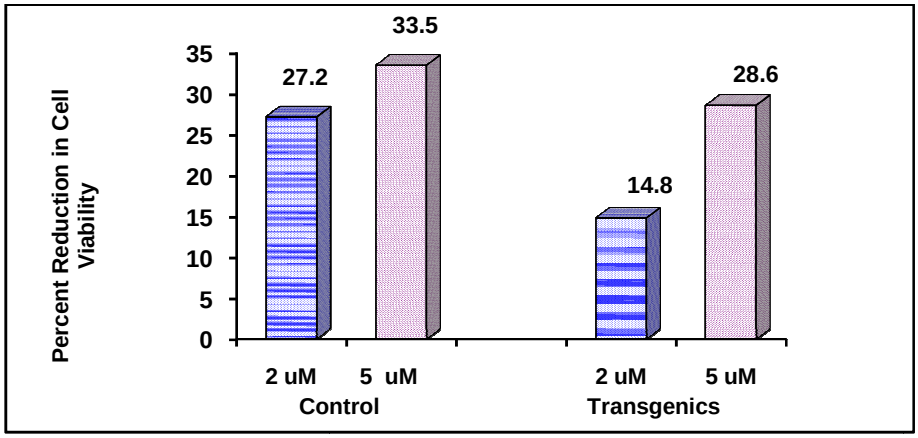

Figure 3: The cell viability in wild type and transgenics over expressing *Ferritin* under Methyl Viologen induced Oxidative Stress.

- **Growth Parameter**

The plant height of same age transgenic and wild type plants showed significant difference. The average plant height of 3 transgenic was127 cms as against 85 cms in control plants (Table 3). Transgenic plants started flowering 80 to 85th days after transferring to pots. But wild type plants initiated flowering only after 105-120th day. The results indicate that there is advancement of flowering by 20-25 days in transgenic plants (Goto *et al.*, 2000).

Table 3: Mean Value of Plant Height Recorded in T_1 Ferritin Transgenics and Wild Type Plants

	Transgenics				Wild type			
Parameter	**T_1**	**T_2**	**T_3**	**Average**	**C_1**	**C_2**	**C_3**	**Average**
Plant Height (cm)	149	123	109	127	78	92	85	85

CONCLUSIONS

The ferritin over expressing plants showed the normal segregation ratio of 3:1 when geminated on kanamycin. The PCR positive plants showed tolerance to MV induced oxidative stress both at seedling level and also at plant level. The percent electrolyte leakage on exposure to MV induced oxidative stress was found to be high in wild type plants compared to transgenic plants. The transgenic plants grew faster and taller and also the flowering time was advanced in transgenic plants compared to wild type plants. The results indicate that the over expression of ferritin in chloroplast gives tolerance against oxidative stress as compared to wild type plants both at seedling level and also at the plant level.

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